AD_____

Award Number: DAMD17-98-1-8199

TITLE: Regulatory Pathways Involved in Heregulin-Induced Proliferation and Differentiation of Human Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Nam Lee
John Koland

CONTRACTING ORGANIZATION: The University of Iowa
Iowa City, Iowa 52242-1320

REPORT DATE: April 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2004	Annual Summary (6 Apr 1998 - 30 Mar 2004)

4. TITLE AND SUBTITLE
Regulatory Pathways Involved in Heregulin-Induced Proliferation and Differentiation of Human Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-98-1-8199

6. AUTHOR(S)
Nam Lee
John Koland

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Iowa
Iowa City, Iowa 52242-1320

E-Mail: Nam-lee@uiowa.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The polypeptide heregulin has been shown to reverse the transformed phenotype of certain cultured breast cancer cell lines and convert them to more normal, milk-producing cells. In contrast, heregulin has been reported to induce proliferation of other breast cancer cell lines. These diverse effects of heregulin are mediated by receptors in the ErbB/HER family, which function as coreceptor complexes. We first attempted to determine how the different signaling pathways activated by ErbB family receptors could elicit alternatively the differentiation or proliferation of breast cancer cells. These experiments were thwarted by an inability of cultured cell models to consistently show heregulin-induced differentiation. Subsequently, we addressed the mechanism of activation of mitogen-activated protein kinase (MAPK) by heregulin coreceptors in breast cancer cells. Interestingly, we observed that inhibitors of the phosphoinositide (PI) 3-kinase signaling pathway blocked the nuclear translocation of MAPK. These findings indicated a point of significant cross-talk between two major signaling pathways activated by heregulin receptors. In our most recent experiments, we examined the dynamics of the ErbB1 (EGF receptor) C-terminal domain, by generating fluorescently labeled EGF receptor proteins. Time-resolved fluorescence anisotropy measurements indicated that the C–terminal domain exhibited significant mobility on the nanosecond time-scale.

14. SUBJECT TERMS
Breast cancer, heregulin, neuregulin, ErbB, differentiation

15. NUMBER OF PAGES
13

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS

INTRODUCTION

Breast cancer cells have been observed to express abnormally high levels of receptor proteins in the ErbB family, which includes the epidermal growth factor (EGF) receptor (ErbB1), ErbB2, ErbB3 and ErbB4 (also designated as HER1-HER4, respectively) (**1-4**). High levels of EGF receptor and ErbB2 expression in tumor cells have been considered indicators of poor prognosis (**5**). Given that these receptors activate mitogenic signaling pathways, it is possible that they play a role in the abnormal proliferation of breast cancer cells. The polypeptide heregulin (**6**) is secreted from breast cancer cells (**7**), and has been shown to activate ErbB2, ErbB3 and ErbB4 receptor proteins (**8-11**). Whereas ErbB4 can respond to heregulin independently, ErbB2 and ErbB3 have been shown to function together as a coreceptor for heregulin (**12**). With the discovery of heregulin (originally designated as Neu differentiation factor) came the observation that this factor could induce the re-differentiation of certain cultured breast cancer cell lines, specifically the cell lines MDA-MB-453 and AU-565 (**13**). Hence, in response to heregulin, these breast cancer cells showed a flatter morphology, the presence of lipid droplets, and elevated levels of the milk protein casein. The observation that heregulin can alternatively induce either the proliferation or the re-differentiation of breast cancer cells raises numerous questions about the mechanisms by which this ErbB receptor ligand activates cellular responses. Presumably, clarifying these cellular control mechanisms would lead to a better understanding of breast cancer development, which in turn could lead to the discovery of novel therapeutic or prophylactic measures. Multiple signaling pathways are engaged by ErbB family receptors in response to heregulin. The focus of the proposed research was to (1) identify those signaling pathways that alternatively elicit either the proliferation or differentiation of breast cancer cells, and (2) investigate the molecular mechanisms by which ErbB receptors activate these distinct signaling pathways.

Explanatory note from the Trainer: The course of this project was interrupted two times by a change in the trainee and appropriate revisions to the statement of work. The first trainee, Ms. Morven Shearer, was a promising graduate student in our pharmacology program. She vigorously pursued the goals of her original statement of work, but unfortunately was frustrated by an inability to consistently reproduce the previously documented heregulin-induced differentiation of cultured breast cancer cells, the mechanism of which was to be examined in the originally proposed research. Ms. Shearer, largely for personal reasons, decided to withdraw from our graduate program and pursue graduate research in her home country of Scotland. Because of some delays by our grants administration offices, no salary support for Ms. Shearer was ever derived from this fellowship award. Subsequently, we requested to replace Ms. Shearer with a new trainee, Mr. Huaming Tan, another graduate student in our department. This request was allowed and a revised statement of work was approved after it was included in the May 2001 progress report. Regrettably, Mr. Tan, although initially enthusiastic about his graduate studies, decided he would withdraw from the program to pursue bioinformatics research in another program. Subsequently, we requested that Mr. Tan be replaced as the trainee by Mr. Nam Lee, a talented graduate student in the University of Iowa Molecular Biology Program of which I am a faculty member. This request was approved as well as a revised statement of work on 2 Jan 02. Mr. Lee has quite successfully pursued this revised statement of work, and with the funds of the fellowship now exhausted, he finds himself on track to complete his Ph.D. studies in the breast cancer research area.

As the Trainer on this predoctoral fellowship, I greatly regret the attrition of the first two trainees that was suffered during the course of its execution. While I necessarily accept responsibility for not identifying a second trainee who would successfully pursue this project, I had truly expected that Mr. Tan, who was an excellent student, was greatly interested in doing so. I was quite surprised when told of his change of research interest and his desire to leave the program. Given these concerns, I am all the more pleased of the progress that Mr. Lee has shown in the course of this project, and I hope that his

training will be viewed as a significant achievement by the Breast Cancer Research Program. In this final progress report, the accomplishments of each of the trainees will be described in turn.

BODY

Trainee Morven C. Shearer:

The goals of the original project were to ascertain the mechanisms by which heregulin, a polypeptide ligand that activates ErbB family receptors, could induce alternatively the proliferation or differentiation of breast cancer cells. To this end, we perfected analytic techniques for assessing the extent of differentiation of cultured breast cancer cell lines, which included the light microscopic "Oil Red O" assay of milk droplet formation and the expression of certain differentiation markers, such as the milk protein casein and the cell surface E-cadherin protein. As detailed in the initial progress report, these assays could discriminate between breast cancer cell lines of differing differentiation status. However, numerous attempts to reproduce the published observation of heregulin-induced breast cancer cell re-differentiation met with limited success, as no consistent changes in these various differentiation markers were seen with heregulin treatment. While it remains possible that yet unknown factors contributed to our inability to reproduce the documented differentiating effects of heregulin, we are left skeptical as to whether the phenomenon is indeed a valid one. Upon requesting assistance from a laboratory that initially published these findings, we were informed that the breast cancer cell line investigated was at the present time contaminated and not available for dissemination.

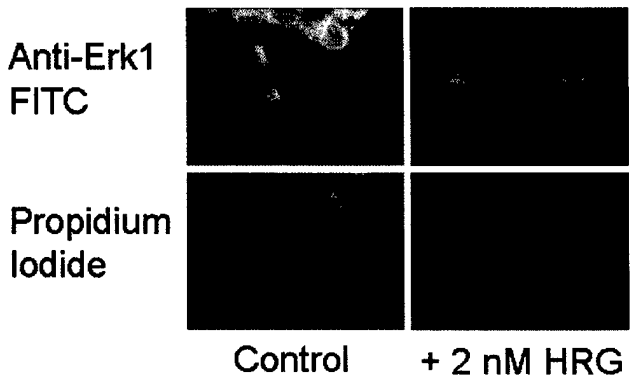

Figure 1. Heregulin-dependent nuclear translocation of MAPK in NIH-3T3-B3 cells. NIH-3T3 fibroblasts expressing endogenous ErbB2 and recombinant ErbB3 proteins (NIH-3T3-B3 cells) were cultured on glass coverslips, then serum-deprived overnight. Cells were subsequently stimulated for 1 h at 37 C with 2 nM heregulin-β1 (HRG) or vehicle (Control) as indicated, and then subjected to immunostaining with MAPK antibody (anti-Erk1) and FITC-conjugated secondary antibody. MAPK localization was assessed by laser confocal microscopy (green staining, upper panels). Nuclei were stained with propidium iodide (red-orange staining, lower panels).

While the initial observation that heregulin can induce a re-differentiation of breast cancer cells is to our mind subject to reappraisal, there is no question that heregulin and ErbB receptor systems contribute to the development of human breast cancer. Numerous recent works have underscored the significance of ErbB receptors in the diagnosis and prognosis of mammary cancers, and indeed an immunotherapeutic agent targeting the ErbB2 (HER2) protein (Herceptin/trastuzumab) has been approved as a new treatment for metastatic breast cancer (**14,15**). Thus, we in the course of this project continued to examine the mechanisms by which ErbB receptors stimulate the proliferation of breast cancer cells.

Trainee Huaming Tan:

With the replacement of the trainee on this project, a new direction of research was pursued as detailed in a revised statement of work. Specifically, we intended to follow-up upon an exciting observation that signaling by ErbB receptors to the mitogen-activated protein kinase (MAPK) signaling pathway depended upon the alternative phosphoinositide (PI) 3-kinase signaling pathway. As both of these pathways have been implicated in cancerous transformation, this previously undiscovered form of signaling "cross-talk" could be of much significance. Figure 1 shows our initial characterization of heregulin-dependent nuclear translocation of MAPK in cultured cells expressing both the ErbB2 and ErbB3 proteins, which together form a functional heregulin coreceptor. Here localization of MAPK

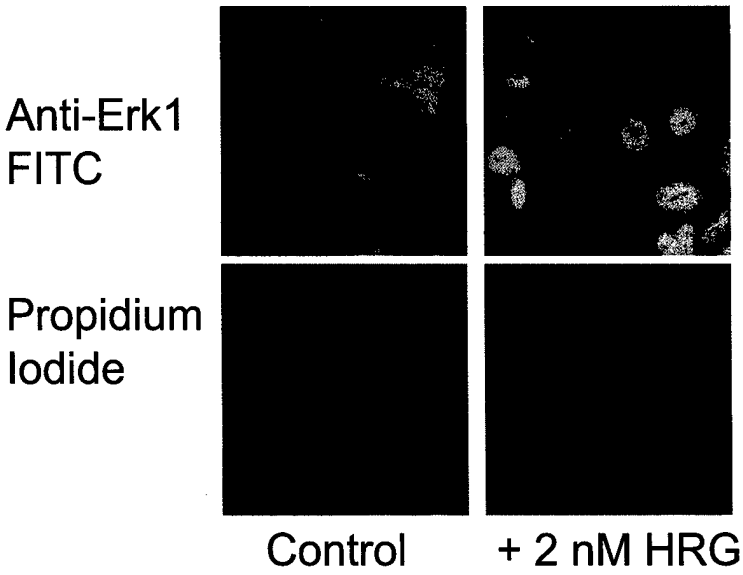

Figure 2. Constitutive localization of MAPK in SK-BR-3 breast cancer cells. SK-BR-3 cells expressing high levels of endogenous ErbB2 and ErbB3 were treated and then analyzed as described in Figure 1. The upper panel shows the heregulin-independent nuclear localization of MAPK, presumably due to constitutive signaling by the endogenous ErbB receptors.

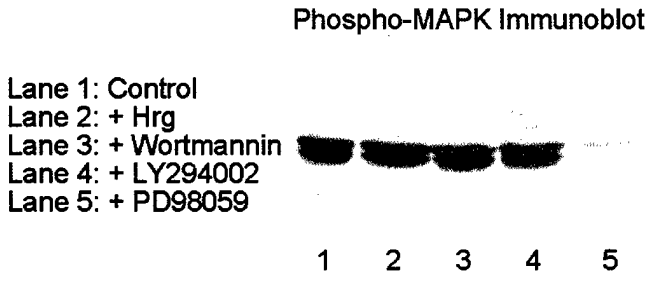

Figure 3. Effects of MAPKK and PI 3-kinase inhibitors on MAPK activation in SK-BR-3 cells. SK-BR-3 breast cancer cells were serum-deprived overnight, then stimulated for 15 min at 37 C with 2 nM heregulin-β1 (+Hrg) or vehicle (Control). Some cells were incubated for 20 min at 37 in the presence of 1 mM wortmannin (nonspecific PI 3-kinase inhibitor), 30 mM LY294002 (PI 3-kinase inhibitor), or 20 mM PD98059 (MAPKK/MEK-1 inhibitor) as indicated. After the various treatments, cells lysates were assayed for MAPK phosphorylation by immunoblotting with a phospho-MAPK antibody (Santa Cruz Biotechnology). Note the constitutive activation of MAPK in SB-BR-3 cells and the failure of PI 3-kinase inhibitors to block MAPK activation.

(Erk1) in cells stained with a Erk1-specific antibody was detected by indirect immunofluorescence microscopy. This phenomenon was characterized in both NIH-3T3 fibroblasts expressing recombinant ErbB receptors, as well as MCF7 breast cancer cells. When a second breast cancer cell line, SK-BR-3 was examined, we observed a constitutive nuclear localization of MAPK that was unaffected by heregulin treatment (Figure 2). Given that SK-BR-3 cells possess exaggerated levels of ErbB2 and ErbB3, this constitutive activation and nuclear localization on MAPK was assumed to reflect a persistent, ligand-independent activation of these endogenous ErbB receptors. It thus appears likely that aberrant MAPK activation contributes to the transformation of at least a subset of mammary tumors.

Because the ErbB3 receptor protein is a particularly proficient recruiter of the PI 3-kinase signaling enzyme, we wished to examine whether the MAPK and PI 3-kinase pathways in any way cooperated in cellular transformation. Thus, we investigated the effects of both MAPK and PI 3-kinase inhibitors on the heregulin-dependent MAPK activation. Interestingly, we found that while the PI 3-kinase inhibitors did not block MAPK activation per se (Figure 3), they did block the nuclear translocation of MAPK that occurs subsequent to its activation (Figure 4). PI 3-kinase inhibitors were found to block both the constitutive nuclear localization of MAPK in SK-BR-3 cells and heregulin-dependent nuclear localization seen in NIH-3T3 cells expressing ErbB2 and ErbB3. This is to our knowledge the first observation of a requirement for PI 3-kinase activity for the translocation of activated MAPK to the nuclear compartment. Because MAPK signaling to transcription factor targets occurs in the cell nucleus, it appears that PI 3-kinase activation, at least in some cellular contexts, is required for transcriptional regulation by MAPK. Thus, the ability of ErbB receptors to activate both the MAPK and PI 3-kinase pathways might allow these pathways to cooperate in signaling to the cell nucleus. One poster abstract describing the work of this trainee was presented at the Era of Hope, Department of Defense Breast Cancer Research Program Meeting in Atlanta, GA in June of 2000.

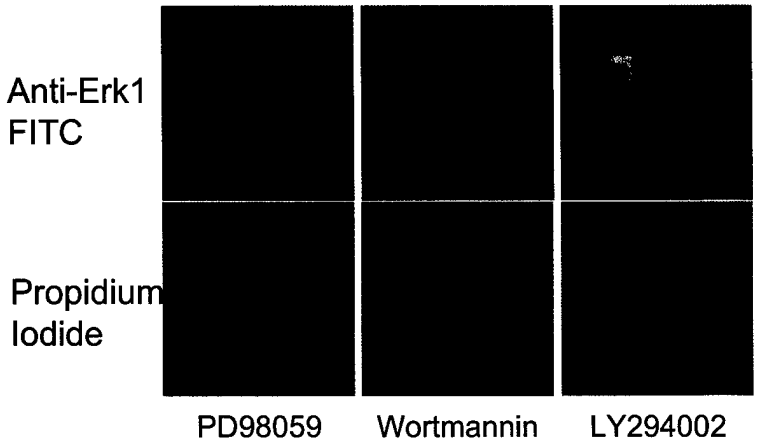

Figure 4. Effect of MAPKK and PI 3-kinase inhibitors on constitutive localization of MAPK SK-BR-3 breast cancer cells. SK-BR-3 cells were cultured on glass coverslips, serum-deprived overnight, then preincubated with either 20 mM PD98059 (MAPKK/MEK-1 inhibitor), 1 mM wortmannin (nonspecific PI 3-kinase inhibitor), or 30 mM LY294002 (PI 3-kinase inhibitor) for 20 min at 37 C. Cells were then immunostained with MAPK antibody and visualized by indirect immunofluorescence microscopy (see Figure 1). MAPK localization (green staining, upper panels). Nuclear staining (red-orange staining, lower panels).

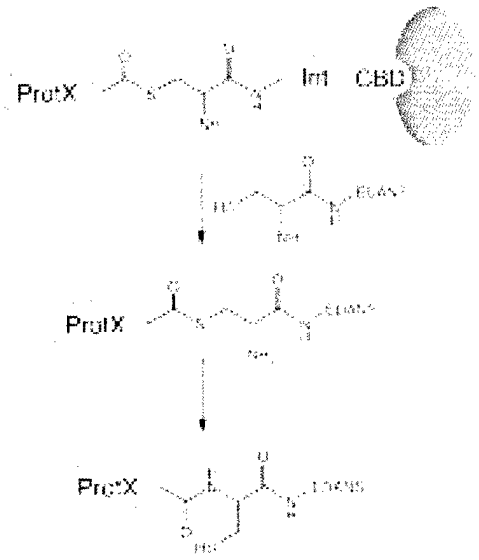

Figure 5. Schematic representation of the intein-based labeling method. The protein of interest (ProtX) is expressed as a tripartite fusion with intein (Int) and chitin binding domain (CBD) sequences. The construct is immobilized on a chitin matrix, and the thiol-ester intermediate is cleaved with an exogenous thiol, here the cysteine conjugate of the fluorophore EDANS, to release purified ProtX. The resulting thiol-ester undergoes an irreversible S-N acyl transition to form a stable amide bond. This method thus generates a purified recombinant protein specifically labeled at its C-terminus with the EDANS fluorophore.

Trainee Nam Y. Lee:

As detailed in a revised statement of work submitted 2 Jan 02, the goals of the last portion of the project were to develop a system for generation of an EGFR (ErbB1) protein that was labeled on its extreme C-terminus with a fluorescent probe molecule and to use this labeled EGFR to analyze the structure and dynamics of the C-terminal phosphorylation domain by fluorescence spectroscopy. These investigations were intended to improve our poor understanding of the mechanisms by which the C-terminal phosphorylation domains of ErbB receptors become phosphorylated and subsequently interact with a diverse set of signaling targets. The various tasks of the statement of work were addressed in turn as described below.

Task 1. Develop methods for purification of recombinant epidermal growth factor receptor (EGFR) proteins site-specifically labeled with fluorescent probe molecules. The strategy here was to employ the "intein-based" method for generation and purification of a recombinant protein labeled at its extreme C-terminus with a fluorescent probe molecule (see Figure 5). While this strategy had been used in the context of an *E. coli* bacterial expression system (**16**), the EGFR is not active when expressed in *E. coli*. Thus, we first needed to engineer a recombinant viral vector with which an intein-fused EGFR protein could be expressed

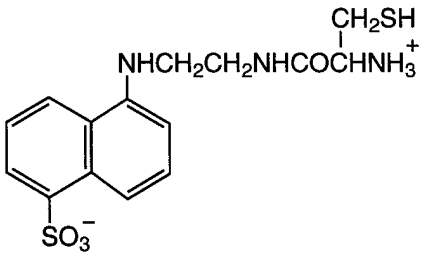

Figure 6. Structure of the EDANS-cysteine conjugate (CEDANS) used in intein-mediated labeling of the EGF receptor C-terminus. The probe was synthesized by the conjugation of 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid (EDANS, Molecular Probes, Inc.) with an N-hydroxysuccinimide ester of cysteine (Novabio-chem) by a modification of a published method.

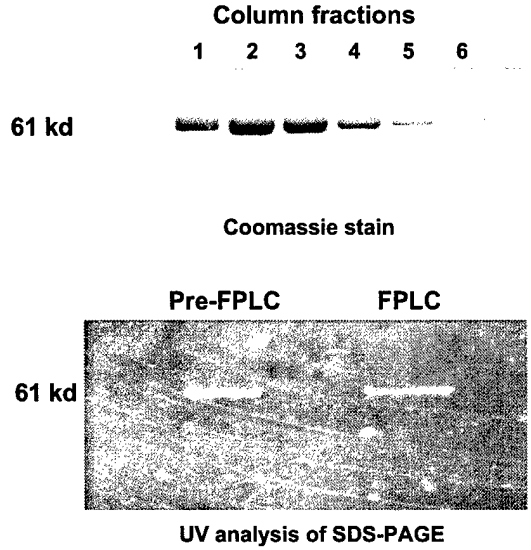

Figure 7. Gel electrophoresis analysis of the recombinant EGFR-CEDANS protein. An EGFR intracellular domain protein was expressed, purified and C-terminally labeled with the intein expression system as adapted to the cultured insect cells. The upper panel (Coomassie Blue protein stain of SDS gel) shows the purity of fractions eluted from the chitin affinity column with the CEDANS conjugate. The lower panel shows the purified conjugate electrophoresed on an SDS gel and visualized with UV illumination. The bright bands are the fluorescence of the EGFR-CEDANS conjugate.

in cultured insect cells. To this end, we successfully subcloned the DNA sequences encoding the chitin-binding domain and intein sequences of the bacterial expression vector pTyb2 (New England Biolabs) into the multiple cloning site of the insect cell expression vector pFastBac1 (Life Technologies). The resulting vector, pFastBac1-Intein, could then be used to express the EGFR intracellular domain coding sequences in cultured insect cells. Furthermore, we could generate an EGFR protein specifically labeled at its C-terminus with a cysteine-fluorophore conjugate of choice. The spectral properties of the fluorophore EDANS were ideal for the proposed investigation. We therefore by modification of a published method (**16**) synthesized its cysteine conjugate (CEDANS) (Figure 6), and used this conjugate in the generation of a labeled EGFR protein (EGFR-CEDANS) (Figure 5).

After some optimization of the intein expression system to make it functional in the context of cultured Sf21 insect cells, we succeeded in generating a purified, C-terminally labeled EGFR conjugate. Figure 7 is a gel electrophoresis analysis of the recombinant EGFR-CEDANS conjugate, which indicates that the protein was effectively purified in good yield (~1 mg) and that the conjugate was indeed fluorescent. Thus, the first task of the statement of work was completed.

Task 2. Fluorescence spectroscopic studies of EGFR C-terminal domain structure and function. The spectral characteristics of the EGFR-CEDANS conjugate were next examined, with the fluorescence excitation and emission spectra (Figure 8) both found to be characteristic of EDANS-derived fluorophores. As anticipated, a good overlap of the CEDANS emission spectrum with the absorbance spectrum of the nucleotide analog TNP-ATP was observed, which suggested that measurements of fluorescence resonance energy transfer (FRET) between the C-terminal CEDANS probe and TNP-ATP bound at the EGFR catalytic site could be used to assess the distance of separation between these sites. While this was one goal of Task 2, we failed to observe significant FRET between the CEDANS conjugate and the bound TNP-ATP analog. Subsequent analysis indicated that the TNP-ATP analog did not bind to the EGFR-CEDANS conjugate as it had been shown to bind to other recombinant EGFR proteins (**17**). The failure of the EGFR-CEDANS conjugate to bind TNP-ATP precluded any assessment of the C-terminus/active site distance. The reasons for the failure of EGFR-CEDANS to bind the nucleotide analog remain unknown.

The second goal of Task 2 was to assess the dynamics of EGFR C-terminal domain motion by time-resolved fluorescence anisotropy studies. These experiments were performed at the Laboratory for Fluorescence Dynamics at the University of Illinois, Urbana, an NIH-funded facility with

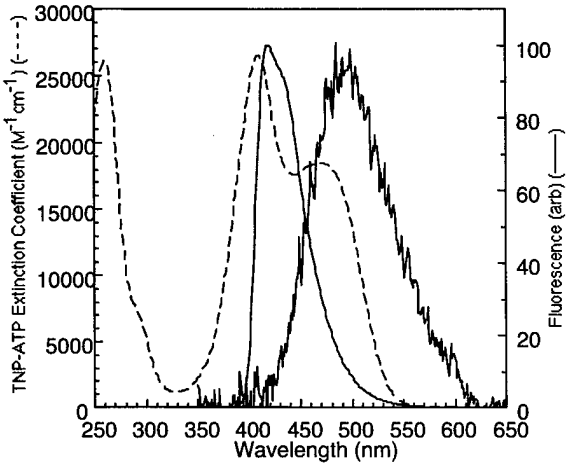

Figure 8. Fluorescene emission spectrum of EGFR-CEDANS. The EGFR-CEDANS conjugate generated with the intein expression system was analyzed by fluorescence spectroscopy. Shown here is the fluorescence emission of the EGFR-CEDANS construct (curve labeled CEDANS), as compared to the absorbance spectrum of the potential energy transfer acceptor TNP-ATP (dashed line). Also shown is the fluorescence emission spectrum of a second EGFR construct generated that incorporated the probe Cascade Blue (Molecular Probes, Inc.).

instrumentation for time-resolved fluorescence spectroscopy. With this instrumentation, the time-dependent decay of the polarization of fluorescence from excited EGFR-CEDANS samples was measured (Figure 9) and the data modeled as a double-exponential relaxation process with characteristic rotational relaxation times and amplitudes for each component (see Table I). (Data were actually recorded in the "frequency domain" using a modulated light source, and were numerically analyzed to yield relaxation times and fractional amplitudes equivalent to those obtained from "time-domain" measurements.)

Two EGFR constructs were analyzed here: the C-terminally full-length EGFR-CEDANS and the C-terminally truncated EGFR-ΔCT-CEDANS, in which the C-terminal phosphorylation domain is deleted and the protein sequence terminates at amino acid 964. Amino acid 964 is the last ordered residue in the published crystal structure of the EGFR cytoplasmic domain and demarks the sequence position at which the mobile C-terminal phosphorylation domain begins. For both constructs, the anisotropy data were well fit by a two-component model (see fitted curves in Figure 9), characterized by two relaxation times and the two fractional amplitudes for the components. The greater of the two relaxation times were 56 and 30 nsec, respectively, for the EGFR-CEDANS and EGFR-ΔCT-CEDANS constructs. These slower components likely reflect the overall tumbling motions of the two molecules, which are roughly of 61 kDa and 38 kDa molecular mass, respectively. Both constructs showed a fast ~0.6 nsec component, which presumably represented the local motion of a small segment of the construct, and indicated that a least some portion of the C-terminal phosphorylation domain was disordered and capable of relatively rapid motion. The amplitude of this fast component was significantly larger for the truncated EGFR-ΔCT-CEDANS construct, which might indicate that the point of truncation, amino acid residue 964, represents a flexible hinge point to which the C-terminal domain is attached. Additional experiments found no significant changes in the anisotropy decay dynamics of the C-terminal domain when it was allowed to autophosphorylate.

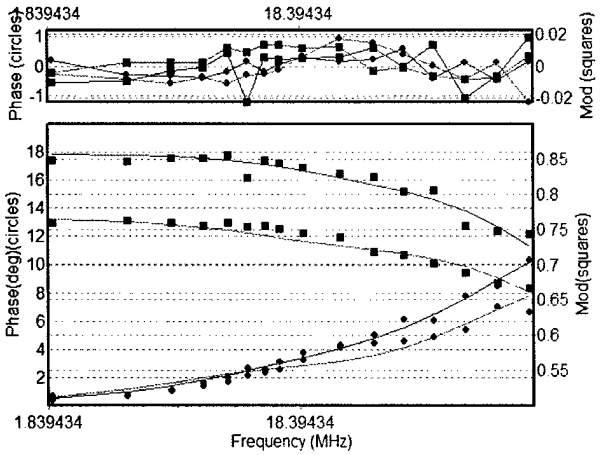

Figure 9. Frequency domain measurements of the fluorescence anisotropy of EGFR-CEDANS conjugates. EGFR-CEDANS conjugates were analyzed in a phase-modulation (frequency domain) fluorescence anisotropy instrument at the Laboratory for Fluorescence Dynamics at the University of Illinois-Urbana. Shown here are the frequency dependencies of the phase delay (◆) and amplitude modulation (■) of the polarization of the fluorescence emission. Two constructs were analyzed, a C-terminally full-length EGFR-CEDANS protein (red symbols) and a C-terminally truncated EGFR-ΔCT-CEDANS protein (blue symbols) (see text).

We interpret our findings to mean that the C-terminal phosphorylation domain of the EGFR, and likely that of other ErbB family members, shows significant motion on the ~1 nsec time-scale. This is significantly faster than the predicted overall tumbling motions of protein of

Table I. Analysis of time-resolved fluorescence anisotropy of EGFR-CEDANS constructs				
Construct	τ_1	f_1	τ_2	f_2
EGFR-CEDANS	56.0 nsec	0.518	0.62 nsec	0.482
EGFR-ΔCT-CEDANS	29.9 nsec	0.279	0.66 nsec	0.721

this size, and likely reflects significant local motion within the C-terminal domain, presumably near the C-terminus and at the point of attachment of the C-terminus to the ordered kinase core (amino acid residue 964). An interesting result was the lack of an effect of phosphorylation on C-terminal domain dynamics. One model that we and others have proposed (**17**) is that the C-terminal domain prior to its phosphorylation interacts with the active site of the kinase, and with its phosphorylation moves from the active site to both interact with signaling targets and allow access of other substrates to the active site. Our results suggest that a static structural model of the EGFR phosphorylation domain might not be valid, and that significant C-terminal domain motion might occur prior to its phosphorylation.

A manuscript describing these biophysical investigations of the EGFR C-terminal domain structure and dynamics is in preparation.

KEY RESEARCH ACCOMPLISHMENTS

Trainee Morven C. Shearer:

- Development and application of the Oil Red-O histochemical method for detection of lipid droplets in cultured breast cancer cells
- Development and application of the Western immunoblotting method for detection of β-casein and E-cadherin expression in cultured breast cancer cells
- Investigation of the potential differentiating effects of heregulin treatment on cultured breast cancer cells by assay of markers of cellular differentiation
- **Trainee Huaming Tan:**
- Demonstration of the constitutive nuclear localization of mitogen-activated protein kinase (MAPK) in some cultured breast cancer cell lines
- Identification of an apparent role for phosphoinositide 3-kinase in the nuclear translocation of the MAPK signaling enzyme
- **Trainee Nam Y. Lee:**
- Development of an intein-based expression system for generation of recombinant proteins in cultured insect cells
- Synthesis of the cysteine conjugates of two fluorescent probe molecules, cysteine-EDANS (CEDANS) and cysteine-Cascade Blue
- Use of the intein-based expression system for the site-specific labeling of the epidermal growth factor receptor (EGFR) C-terminus with fluorescent probe conjugates
- Characterization of the fluorescence of labeled EGFR constructs
- Use of time-resolved fluorescence anisotropy measurements to determine the dynamics of EGFR C-terminal domain motion

REPORTABLE OUTCOMES

Abstract presented:

Huaming Tan, Myong-Soo Kim, and John Koland. Heregulin/ErbB Receptor Signaling in Breast Cancer Cells. Era of Hope, Department of Defense Breast Cancer Research Program Meeting, June 2000, Atlanta, GA.

CONCLUSIONS

Several significant conclusions were made in the course of this project. Our initial studies of the heregulin-dependent re-differentiation of breast cancer cells have led us to conclude that this phenomenon is not so reproducibly observed as suggested by earlier literature reports. This might be because the phenomenon is dependent upon certain experimental factors that remain to be identified, such as subtle variations in culture medium, the exact original of the heregulin preparations, etc. We failed however to identify such experimental factors that would allow us to reproduce the documented phenomenon. Our investigations of the mechanisms of MAPK activation by the ErbB receptor proteins led us to the conclusion that PI 3-kinase activity is required for the nuclear translocation of MAPK that occurs subsequent to its activation. How exactly PI 3-kinase is involved in MAPK translocation and whether this role for PI 3-kinase would be observed more generally in the context of other cell types and receptor systems are important questions for future research. As PI 3-kinase and MAPK can both participate in cellular transformation, the discovery of their interaction in signaling in breast cancer cells could be a significant one in terms of cancer cell biology. We also observed that MAPK is constitutive activated and nuclear localized in at least one breast cancer cell line that expressed abnormally high ErbB receptors levels. This observation could ultimately be important from a clinical standpoint. Specifically, while the activation status of MAPK has been considered as a diagnostic indicator of cellular transformation, our findings indicate that the subcellular localization of MAPK (i.e. either cytoplasmic or nuclear localization should also be considered as a potential diagnostic/prognostic indicator of mammary tumors. Screening of tumor biopsies for the prevalence of nuclear MAPK could thus possibly be a diagnostic/prognostic indicator analogous to ErbB2/HER2 expression levels. Our biophysical studies of the EGFR C-terminal domain dynamics have led us to conclude that the extreme C-terminus and also the point at which the C-terminus attaches to the kinase domain core of the receptor show significant mobility on the nanosecond time scale. This indicates that the C-terminal phosphorylation domain is not immobilized in the active site prior to receptor activation and that the C-terminal phosphorylation domain might rather be always relatively mobile. It is unknown what the exact biologic or clinical significance of our findings here might be. However, a better understanding of the structure and dynamics of the C-terminal domains of ErbB receptor proteins represents another step in our clarification of the signaling mechanisms of these important receptor types.

REFERENCES

1. Kraus, M. H., Issing, W., Miki, T., Popescu, N. C., and Aaronson, S. A. (1989) Isolation and characterization of *ERBB3*, a third member of the *ERBB*/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. *Proc. Natl. Acad. Sci. USA* **86**, 9193-9197
2. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. F. (1989) Studies of the HER-2/*neu* Proto-oncogene in Human Breast and Ovarian Cancer. *Science* **244**, 707-712
3. Lovekin, C., Ellis, I. O., Locker, A., Robertson, J., Bell, J., Nicholson, R., Gullick, W. J., Elston, C. W., and Blamey, R. W. (1991) c-erbB-2 oncoprotein expression in primary and advanced breast cancer. *Br. J. Cancer* **63**, 439-443
4. Gullick, W. J. (1990) 4. The role of the epidermal growth factor receptor and the c-erbB-2 protein in breast cancer. *Int. J. Cancer* **46**, 55-61
5. Jain, S., Filipe, M. I., Gullick, W. J., Linehan, J., and Morris, R. W. (1991) c-erbB-2 proto-oncogene expression and its relationship to survival in gastric carcinoma: An immunohistochemical study on archival material. *Int. J. Cancer* **48**, 668-671
6. Ben-Baruch, N., and Yarden, Y. (1994) Neu differentiation factors: A family of alternatively spliced neuronal and mesenchymal factors. *Proc. Soc. Exp. Biol .Med.* **206**, 221-227
7. Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, H. M., Kuang, W. J., Wood, W. I., Goeddel, D. V., and Vandlen, R. L. (1992) Identification of heregulin, a specific activator of $p185^{erbB2}$. *Science* **256**, 1205-1210
8. Plowman, G. D., Green, J. M., Culouscou, J. M., Carlton, G. W., Rothwell, V. M., and Buckley, S. (1993) Heregulin induces tyrosine phosphorylation of HER4/$p180^{erbB4}$. *Nature* **366**, 473-475
9. Carraway, K. L., III, and Cantley, L. C. (1994) A neu acquaintance for ErbB3 and ErbB4: A role for receptor heterodimerization in growth signaling. *Cell* **78**, 5-8
10. Carraway, K. L., III, Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C., and Cerione, R. A. (1994) The *erbB3* gene product is a receptor for heregulin. *J. Biol. Chem.* **269**, 14303-14306
11. Soltoff, S. P., Carraway, K. L., III, Prigent, S. A., Gullick, W. G., and Cantley, L. C. (1994) ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. *Mol. Cell. Biol.* **14**, 3550-3558
12. Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., III. (1994) Coexpression of *erb*B2 and *erb*B3 proteins reconstitutes a high affinity receptor for heregulin. *J. Biol. Chem.* **269**, 14661-14665
13. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B., Koski, R. A., Lu, H. S., and Yarden, Y. (1992) Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. *Cell* **69**, 559-572
14. de Bono, J. S., and Rowinsky, E. K. (2002) The ErbB receptor family: a therapeutic target for cancer. *Trends in Molecular Medicine* **8**, S19-26
15. Stebbing, J., Copson, E., and O'Reilly, S. (2000) Herceptin (trastuzamab) in advanced breast cancer. *Cancer Treatment Rev.* **26**, 287-290
16. Mukhopadhyay, J., Kapanidis, A. N., Mekler, V., Kortkhonjia, E., Ebright, Y. W., and Ebright, R. H. (2001) Translocation of sigma(70) with RNA polymerase during transcription: fluorescence resonance energy transfer assay for movement relative to DNA. *Cell* **106**, 453-463

17. Cheng, K. R., and Koland, J. G. (1996) Nucleotide binding by the epidermal growth factor receptor protein-tyrosine kinase: trinitrophenyl-ATP as a spectroscopic probe. *J. Biol. Chem.* **271**, 311-318

PERSONNEL RECEIVING PAY FROM THE AWARD

1. Huaming Tan

2. Nam Y. Lee